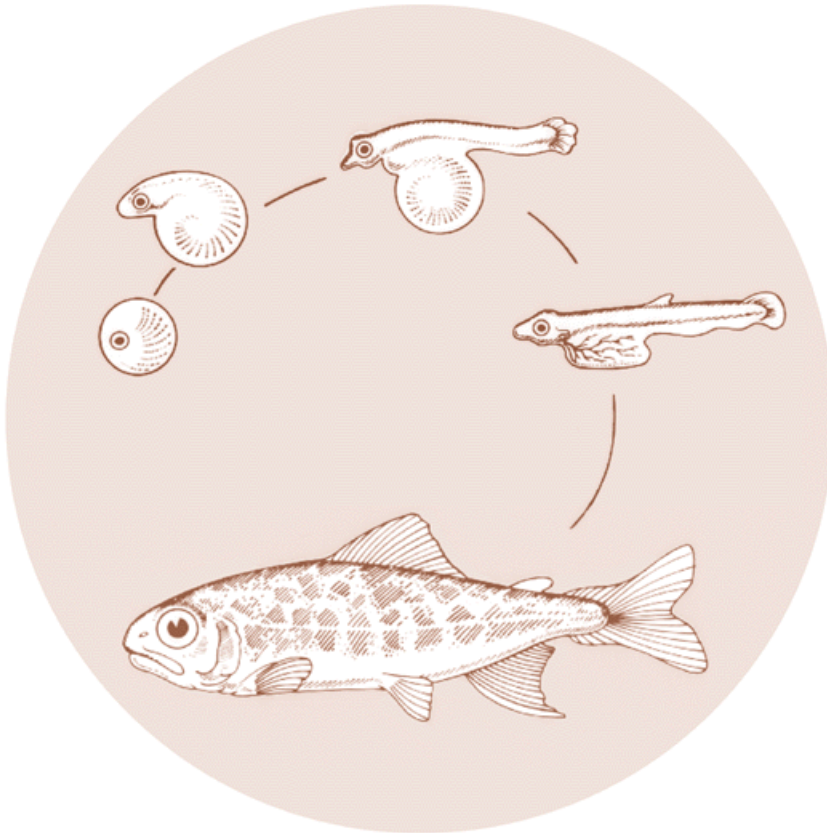


July 1990

# RESEARCH TO IDENTIFY EFFECTIVE ANITFUNGAL AGENTS

Annual Report



DOE/BP-02737-1



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# RESEARCH TO IDENTIFY EFFECTIVE ANTIFUNGAL AGENTS

## Annual Report

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## Abstract

Malachite green is effective in control of fungus on fish and fish eggs, but due to suspected teratogenicity its use is limited to the treatment of nonfood fish under an Investigational New Animal Drug Application held by the U.S. Fish and Wildlife Service. The reregistration of malachite green for treatment of fungus on food fish is highly unlikely. Consequently, the search for safe and effective aquatic fungicides must continue. The objectives of this study involved the selection and evaluation of candidate antifungal compounds on cultured fungus (Saprolegniales), fungus-infected eggs of rainbow trout and chinook salmon, and finally on adult spring chinook salmon. Minimum effective concentrations *in vivo* and minimum safe concentrations were established in order to calculate safety indices. Safety indices and hatching success were then used to evaluate effectiveness of candidate antifungal compounds. The four compounds that demonstrated the greatest safety on eggs were A-73336 (>4), Diquat (>5), formalin (5), and Herbisan (>5). Candidates A-39909, dichlorophen, 8-quinolinol sulfate, and peptides lacked effectiveness for control of fungus or were toxic to the eggs at fungicidal concentrations. Potassium permanganate will be tested further to reassess its potential.

The adult rearing facility was constructed and tested. Test groups of adult spring chinook were established to evaluate the extent of fungal infections at the site and the prophylactic effects of malachite green and formalin as standards against which to compare candidate compounds. These tests are ongoing.

## **Introduction**

Aquatic fungi (Saprolegniales) are ubiquitous in natural water supplies of fish hatcheries often causing serious disease problems. Malachite green is effective in control of fungus on fish and fish eggs, but due to suspected teratogenicity (Meyer and Jorgenson 1984) its use is limited to the treatment of non-food fish (i.e., eggs or adult salmon held for spawning) under an Investigational New Animal Drug Application held by the U.S. Fish and Wildlife Service. The reregistration of malachite green for treatment of fungus on food fish is highly unlikely. Presently, there is one registered aquatic fungicide, formalin, but it is not completely effective in control of fungus on fish or their eggs. Consequently, the search for safe and effective aquatic fungicides must continue.

Bailey (1984) and Bailey and Jeffrey (1989) reported results of tests with over 200 compounds that were chosen for fungicidal activity. More than half were found to be unsuitable as aquatic fungicides in preliminary tests because of their lack of activity against fungi, toxicity to fish or their eggs, insolubility in water, or potential carcinogenicity. However, several of the better candidates showed potential for control of fungus on fish eggs and were chosen for further evaluation.

The objectives of the present contract study were to select and evaluate up to 10 candidate fungicides. Evaluations for Goal I involve laboratory studies on efficacy of candidate compounds on cultured Saprolegniales, and on eggs of rainbow trout and chinook salmon that were previously infected with the fungus. Candidates that are effective

for control of fungus on eggs will be tested further on adult spring chinook salmon (Goal II).

The main objective this year for Goal II was to determine the degree of fungal infection in untreated, control fish to establish the extent of the problem. Secondly, we needed to evaluate the prophylactic effects of malachite green and formalin against which to judge subsequent testing of candidate compounds.

## **Materials and Methods**

### **Goal I. Eggs and Juveniles**

#### **In Vitro Tests**

Pure strains of aquatic fungi were obtained from the American Type Culture Collection (ATCC). *Saprolegnia hypogyna* (ATCC 28275) was used for both range finding and minimum inhibitory concentration tests. Test procedures used were those developed by Bailey (1983a,b). The method involves an in *vitro* screening technique modified from that of Golden and Oster (1947) and a minimum inhibitory concentration determination based on the percent inhibition of growth in diameter of colonies (Bailey and Jeffrey 1989).

Range finding--Standard petri dishes were filled with 20 mL of corn meal agar and inoculated with agar plugs augmented with fungi (5 mm in diameter). The fungi was allowed to incubate at 20°C for approximately 96 hours. Agar plugs were removed from the edge of the colonies with a #1 cork borer. Stock solutions of chemicals were prepared to achieve final concentrations of 1, 10, and 100 mg/L. The depressions of Coors porcelain spot plates were filled with three

replicates of each of the candidate chemicals and the solvent, positive, and negative controls.

Agar plugs were then added to the depressions of the spot plates for exposures of 15 and 60 minutes. Agar plugs were removed from the spot plates, rinsed three times with sterile distilled water, and placed on tri-petri dishes containing 30 mL of corn meal agar. Cultures were incubated in continuous light inside an environmental control chamber maintained at 20°C (+ 2°C). The plates were examined for mycelial growth after 48, 96, and 168 hours of incubation.

Minimum inhibitor-v concentration--Inoculations, incubation, and stock solution preparation were as stated above. Agar plugs were removed from the edge of the fungal colonies as previously described and exposed in triplicate to five delineative concentrations between 0.1 and 1.0 mg/L, 1.0 and 10.0 mg/L, or 10.0 and 100.0 mg/L, depending on the activity observed in the range-finding test.

The agar plugs were exposed to the test chemicals in triplicate for 15 and 60 minutes. They were rinsed with sterile distilled water and placed on standard petri dishes containing 10 mL of corn meal agar. Cultures were incubated in a lighted environmental control chamber at 20°C for 48 hours, and the colony diameters were measured with a vernier caliper.

#### In Vivo Tests

Green eggs from Trout Lodge (McMillin. Washington) were placed in Heath incubating trays (500 eggs per tray) with the use of a modified egg counting board. Characteristics of the well water used

for incubation were a total hardness of 138 mg/L as  $\text{CaCO}_3$ , alkalinity of 105 mg/L as  $\text{CaCO}_3$ , pH of 8.0, and temperature of 12°C.

The eggs were confined within a 6-inch diameter acrylic ring that was 1-inch in height and attached with silicone to the screen of each incubator tray. Two trays were used as replicates for each treatment level. All trays (except the negative controls) were inoculated with *S. ferax* on hemp seeds suspended by a tea ball in each of the trays. We allowed the eggs to become fungused for approximately 7 days. The chemicals were delivered to the back of the egg hatching trays with the use of a peristaltic pump to achieve a specific desired concentration. Eggs were exposed to the fungicides for 15 or 60 minutes. Treatments were administered three times weekly for a period of 2 weeks. Mortality and fungal infection were assessed prior to the first treatment (pretreatment), after the last treatment (post-treatment), and after the eggs hatched (post-hatch). Infection rates (% increase) were calculated by subtracting pretreatment infection rates from post-treatment rates. Total hatch was assessed by subtracting the pretreatment mortality from the total number hatched.

### Toxicity Testing

Toxicity of the candidate fungicides was established in dip treatments with green and eyed eggs of rainbow trout. Twenty-five eggs were placed in 125-mL glass jars containing polyurethane screen mesh bottoms that were suspended in 12.0 ± 1.0°C well water. The green eggs were dipped three times a week for 2 weeks into a dilution series (2.5-L jars) of each test chemical for 15 or 60 minutes and then



returned to well water. Eyed eggs were treated in a similar manner except fewer dip treatments were done due to egg hatching.

The dilution series was generally based on a use pattern of 1/2X, 1X, 3X, and 5X. The 1X concentration was the concentration we felt would be effective for control of fungus. Mortality observations were taken daily and at the end of the test and egg hatching success was recorded. Margins of safety for each chemical were established by dividing the toxic concentration by the effective concentration for respective exposure times.

## **Goal II. Adults**

An adult holding system was constructed adjacent to the Oregon Cooperative Fishery Research Unit's Smith Farm Experimental Hatchery in Corvallis, Oregon. The system was tested by transporting 25 adult spring chinook from the Oregon Department of Fish and Wildlife's Dexter Holding Facility to our new holding system on 3 July 1990. The fish performed well and no mortalities were experienced.

On 10 July 1990, 75 adult spring chinook from Dexter Holding Facility were trucked to the new holding system. These fish were injected with antibiotic (erythromycin and oxolinic acid) the day of transport. On July 20, 1990, the fish were distributed in equal numbers (n=10) among six 10-foot diameter by 4-foot deep tanks.

Treatment protocols for malachite green and formalin were established following consultation with state and federal pathologists and hatchery managers. The protocols selected were those currently used at Willamette Hatchery for formalin or recommended by state

pathologists for malachite green because we wanted to simulate as closely as possible treatments used at production facilities.

This particular hatchery is most appropriate for our stock of fish. Treatment commenced on 22 July 1990 and consisted of treating fish with a bolus every other day of 0.5 ppm Malachite green or 1:6000 formalin in the flow-through system. The flow rate was about 45 L/min for each tank. Dye depletion studies with fluorescein and others with malachite green using fluorometry and spectrophotometry, respectively, indicated effective treatment levels of the chemicals would be 2-4 hours.

The study design was established after consultation with research pathologists and a statistician. All test groups, controls, malachite green and formalin, were duplicated. Mortality was followed through time of maturity in all experimental groups.

### **Modifications in Test Procedures**

Several modifications in test procedures were instituted for subsequent experiments as a result of negotiations with BPA staff and some erratic test results. Toxicity tests in the future will be done in the egg incubators simultaneously with the efficacy exposures. Therefore, uninfected eggs from the same source will also be exposed to the selected efficacious concentration as well as at the 3X and 5X rates. To ensure better mixing of candidate toxicants in the incubation water, a single initial tray was devoted to mixing by installing baffles in the tray to disperse the toxicant.

## **Results and Discussion**

The infection rate of healthy rainbow trout eggs varied between groups of eggs and between eggs of the same group for different chemicals (Appendices 1-5). Initial infection rate for eggs in the first test (Appendix 1) was very low: however, the increase in fungal infection in the positive control was substantially higher at 43.7% than in the negative control at 18.2%. Those eggs treated with A-73336 and Diquat showed good hatch percentages while those treated with 8-quinolinol sulfate showed poorer hatch. Since the initial infection rate was low on these tests, the compounds were tested again.

The initial infection rate was higher in subsequent tests (Appendices 2-5) in which we attempted to get an infection rate of about 10%. In those exposures A-73336 at 5 mg/L demonstrated better hatch percentage than other candidates and also better than with malachite green. Eggs exposed to Diquat also showed greater hatch percentages. Herbisan 5 (Appendix 5) showed improved hatching percentages at 10 mg/L in 15-minutes exposures but less efficiency in the longer exposure and at higher concentrations. We noticed precipitation at higher concentrations: probably because the chemical was formulated in xylene. We plan to test another sample of technical grade Herbisan 5.

Minimum effective concentrations (MEC) and minimum safe concentrations (MSC) were estimated from exposure data (Appendix 7) and often provided only “greater than” information (Table 1). Therefore, some of the values are not precise, but they are useful in preliminary evaluations. The four compounds demonstrating the

greatest safety are A-73336 (>4), Diquat (25), formalin (5), and Herbisan (25). Candidates A-39909, dichlorophen, 8-quinolinol sulfate, and peptides lack effectiveness for control of fungus or are toxic to the eggs at fungicidal concentrations. The latter candidates will not be considered further in the efforts to find an effective fungicide for use on fish and their eggs. Potassium permanganate will be tested further to reassess its potential.

The initial study of adults was completed on 11 September 1990. At that time, all animals in both control tanks had died, whereas 10% or less mortality was suffered in the groups treated with malachite green or formalin (Table II). Examination of all animals on 14 August 1990 (approximately 1 month after arrival at the site) revealed the incidence of fungal growth on the gills of control fish, but no occurrence of fungal growth on the gills of any treated fish.

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Table 1. Effectiveness (mg/L) of candidate fungicides on cultured fungus (*Saprolegnia hypogyna*) and on eyed eggs of rainbow trout infected with fungus in terms of MIC (minimum inhibitory concentration), MEC (minimum effective concentration). and MSC (minimum safe concentration).

| Chemical name                | Exposure time (min) | <i>In vitro</i> |      | <i>In vivo</i> MEC | Toxicity (MSC) eyed eggs | Safety index eyed eggs |
|------------------------------|---------------------|-----------------|------|--------------------|--------------------------|------------------------|
|                              |                     | Range           | MIC  |                    |                          |                        |
| A-39909                      | 15                  | >1<10           | ≤1.0 | >5.0               | 5.0                      | <1                     |
|                              | 60                  | >1<10           | ≤1.0 | >5.0               | 2.5                      | >0.5                   |
| A-73336                      | 15                  | >1<10           | ≤1.0 | >5.0               | ≥20                      | 24                     |
|                              | 60                  | >1<10           | ≤1.0 | >5.0               | ≥20                      | 24                     |
| Dichlorophen                 | 15                  | >1<10           | <5.0 | >5.0               | 210                      | 2                      |
|                              | 60                  | >1<10           | <3.0 | >5.0               | 210                      | 2                      |
| Diquat                       | 15                  | >100            | <700 | 100                | ≥500                     | 25                     |
|                              | 60                  | >100            | <500 | 100                | ≥500                     | 25                     |
| Formalin                     | 15                  | >100            | <300 | 1667               | 5,000                    | 3                      |
|                              | 60                  | >100            | <300 | 1000               | 1,667                    | 1.7                    |
| Herbisan                     | 15                  | >100            | --   | 210                | ≥50                      | 25                     |
|                              | 60                  | >100            | --   | ≥10                | ≥50                      | ≥5                     |
| KMnO <sub>4</sub>            | 15                  | >100            | <500 | 100                | ≥250                     | >2.5                   |
|                              | 60                  | >100            | <250 | 100                | 150                      | 1.5                    |
| Malachite green              | 15                  | <1              | 0.5  | 5.0                | ≥25                      | ≥5                     |
|                              | 60                  | <1              | 0.5  | 5.0                | 15                       | 3                      |
| Peptides                     | 15                  | >100            | --   | --                 | --                       | --                     |
|                              | 60                  | >100            | --   | --                 | --                       | --                     |
| 8-Quinolinol SO <sub>4</sub> | 15                  | >100            | ≤100 | >100               | ≥250                     | <2.5                   |
|                              | 60                  | >100            | ≤100 | >100               | 150                      | <1.5                   |

Table II. Mortality, Maturation, and Incidence of Fungal Growth in Adult Spring Chinook salmon held in Corvallis, Oregon.

| Treatment       | n  | %mortality | %maturity | %fungal growth |
|-----------------|----|------------|-----------|----------------|
| Control         | 11 | 100        | 0         | 100 (1)        |
| Control         | 10 | 100        | 0         | 33 (6)         |
| Malachite Green | 10 | 10         | 10        | 0 (10)         |
| Malachite Green | 10 | 10         | 50        | 0 (10)         |
| Formalin        | 10 | 0          | 30        | 0 (10)         |
| Formalin        | 10 | 0          | 40        | 0 (10)         |

Key: "n" indicates the number of individuals at the beginning of the experiment; "% mortality" indicates the percentage of "n" that died as of 11 September 1990; "% maturity" indicates the percentage of "n" that reached sexual ripeness as of 20 September 1990; "% fungal growth" indicates the percentage of surviving individuals (given in parentheses) with fungal growth on gills on 14 August 1990.

## Appendices



Appendix 1. Effectiveness of candidate fungicides on infected eggs of rainbow trout.

| Compound         | Concentration (mg/L) | Exposure (min) | infection (%) |          | Hatch <sup>a</sup> (%) |
|------------------|----------------------|----------------|---------------|----------|------------------------|
|                  |                      |                | Initial       | Increase |                        |
| (+) Control      | --                   |                | 1.3           | 43.7     | 68.3                   |
| (-) Control      | --                   |                | 0.0           | 18.2     | 85.8                   |
| A-73336          | 2.0                  | 15             | 0.1           | 24.0     | 85.1                   |
| A-73336          | 2.0                  | 60             | 1.0           | 12.2     | 89.2                   |
| A-73336          | 4.0                  | 15             | 0.2           | 17.8     | 93.3                   |
| A-73336          | 4.0                  | 60             | 1.0           | 7.9      | 88.8                   |
| 8-Quinolinol SO4 | 50.0                 | 15             | 0.0           | 45.1     | 62.4                   |
| 8-Quinolinol SO4 | 50.0                 | 60             | 2.3           | 39.4     | 68.6                   |
| Diquat           | 50.0                 | 15             | 1.3           | 32.8     | 85.2                   |
| Diquat           | 50.0                 | 60             | 3.6           | 25.0     | 87.9                   |
| Diquat           | 100.0                | 15             | 0.7           | 31.8     | 83.4                   |
| Diquat           | 100.0                | 60             | 1.9           | 24.1     | 75.0                   |

<sup>a</sup>Hatch calculation:  $\frac{\text{number hatched}}{\text{total} - \text{initial morts}} \times 100$

Appendix 2. Effectiveness of candidate fungicides on infected eggs of rainbow trout.

| Compound                     | Concentration (mg/L) | Exposure (min) | Infection (%) |          | Hatch <sup>a</sup> (%) |
|------------------------------|----------------------|----------------|---------------|----------|------------------------|
|                              |                      |                | Initial       | Increase |                        |
| (+) Control                  | --                   | --             | 9.8           | 53.9     | 30.6                   |
| (-) Control                  | --                   | --             | 1.9           | 70.6     | 27.7                   |
| Malachite green              | 5.0                  | 15             | 9.0           | 27.4     | 68.6                   |
| Malachite green              | 5.0                  | 60             | 10.9          | 26.7     | 80.8                   |
| A-73336                      | 5.0                  | 15             | 9.8           | 14.9     | 85.0                   |
| A-73336                      | 5.0                  | 60             | 10.9          | 6.7      | 88.1                   |
| 8-Quinolinol SO <sub>4</sub> | 100.0                | 15             | 3.0           | 63.6     | 35.3                   |
| 8-Quinolinol SO <sub>4</sub> | 100.0                | 60             | 14.8          | 51.5     | 28.1                   |
| Dichlorophen                 | 2.0                  | 15             | 10.4          | 48.4     | 47.8                   |
| Dichlorophen                 | 2.0                  | 60             | 1.4           | 45.5     | 58.0                   |
| Dichlorophen                 | 5.0                  | 15             | 6.6           | 35.0     | 62.6                   |
| Dichlorophen                 | 5.0                  | 60             | 5.9           | 45.6     | 70.1                   |

<sup>a</sup>Hatch calculation:  $\frac{\text{total number hatched}}{\text{total} - \text{initial morts}} \times 100$

Appendix 3. Effectiveness of candidate fungicides on infected eggs of rainbow trout .

| Compound          | Concentration (mg/L) | Exposure (min) | Infection (%) |          | Hatch <sup>a</sup> (%) |
|-------------------|----------------------|----------------|---------------|----------|------------------------|
|                   |                      |                | Initial       | Increase |                        |
| (+)Control        |                      |                | 17.4          | 43.5     | 34.6                   |
| ( - )Control      |                      |                | 0.4           | 24.9     | 70 .1                  |
| Malachite green   | 5.0                  | 15             | 5.2           | 33.1     | 56.6                   |
| Malachite green   | 5.0                  | 60             | 9.0           | 24.4     | 62.2                   |
| KMnO <sub>4</sub> | 50.0                 | 15             | 7 .1          | 46 .1    | 38.3                   |
| KMnO <sub>4</sub> | 50.0                 | 60             | 14.0          | 35.6     | 37.1                   |
| KMnO <sub>4</sub> | 100.0                | 15             | 5.6           | 37.4     | 57.7                   |
| KMnO <sub>4</sub> | 100.0                | 60             | 14.6          | 25.0     | 44.5                   |
| Formalin          | 100.0                | 15             | 9.6           | 48.1     | 26.0                   |
| Formalin          | 100.0                | 60             | 10.1          | 37.3     | 38.1                   |
| Formalin          | 250.0                | 15             | 12.3          | 38.4     | 34.2                   |
| Formalin          | 250.0                | 60             | 6.5           | 36.5     | 40.5                   |

<sup>a</sup>Hatch calculation:  $\frac{\text{number hatched}}{\text{total} - \text{initial morts}} \times 100$

Appendix 4. Effectiveness of candidate fungicides on infected eggs of rainbow trout.

| Compound    | Concentration (mg/L) | Exposure (min) | Infection (%) |          | Hatch <sup>a</sup> (%) |
|-------------|----------------------|----------------|---------------|----------|------------------------|
|             |                      |                | Initial       | Increase |                        |
| (+) Control |                      |                | 7.6           | 42.7     | 44.0                   |
| (-) Control |                      |                | 1.5           | 36.4     | 62.8                   |
| A-39909     | 2.5                  | 15             | 10.2          | 30.3     | 46.3                   |
| A-39909     | 2.5                  | 60             | 5.7           | 31.4     | 53.9                   |
| A-39909     | 5.0                  | 15             | 9.8           | 37.1     | 55.5                   |
| A-39909     | 5.0                  | 60             | 6.5           | 70.0     | 19.2                   |
| Diquat      | 50.0                 | 15             | 14.9          | 33.8     | 66.1                   |
| Diquat      | 50.0                 | 60             | 7.2           | 40.2     | 59.2                   |
| Diquat      | 100.0                | 15             | 8.0           | 23.8     | 71.9                   |
| Diquat      | 100.0                | 60             | 8.5           | 26.9     | 68.2                   |
| Diquat      | 200.0                | 15             | 11.8          | 41.8     | 52.5                   |
| Diquat      | 200.0                | 60             | 7.5           | 32.0     | 71.1                   |

<sup>a</sup>Hatch calculation:  $\frac{\text{number hatched}}{\text{total initial morts}} \times 100$

Appendix 5. Effectiveness of Herbisan 5 on infected eggs of rainbow trout.

| Compound    | Concentration (mg/L) | Exposure (min) | Infection (%) |          | Hatch <sup>a</sup> (%) |
|-------------|----------------------|----------------|---------------|----------|------------------------|
|             |                      |                | Initial       | Increase |                        |
| (+) Control | --                   |                | 13.9          | 31.5     | 58.3                   |
| (-) Control | --                   |                | 1.4           | 30.0     | 65.5                   |
| Herbisan 5  | 1.0                  | 15             | 14.2          | 35.5     | 55.0                   |
| Herbisan 5  | 1.0                  | 60             | 9.9           | 22.4     | 64.8                   |
| Herbisan 5  | 5.0                  | 15             | 14.2          | 23.3     | 54.7                   |
| Herbisan 5  | 5.0                  | 60             | 13.3          | 23.2     | 58.7                   |
| Herbisan 5  | 10.0                 | 15             | 6.9           | 25.6     | 71.0                   |
| Herbisan 5  | 10.0                 | 60             | 10.3          | 54.4     | 36.5                   |
| Herbisan 5  | 50.0                 | 15             | 18.8          | 33.1     | 44.8                   |
| Herbisan 5  | 50.0                 | 60             | 16.4          | 58.2     | 16.8                   |
| Herbisan 5  | 100.0                | 15             | 13.6          | 50.6     | 27.4                   |
| Herbisan 5  | 100.0                | 60             | 10.5          | 68.4     | 2.9                    |

<sup>a</sup>Hatch calculation:  $\frac{\text{number hatched}}{\text{total} - \text{initial morts}} \times 100$

Appendix 6. Effectiveness of Formalin on infected eggs of rainbow trout.

| Compound    | Concentration (mg/L) | Exposure (min) | Infection (%) |          | Hatch <sup>a</sup> (%) |
|-------------|----------------------|----------------|---------------|----------|------------------------|
|             |                      |                | Initial       | Increase |                        |
| (+) Control | --                   | --             | 35.2          | 38.6     | 48.4                   |
| (-) Control | --                   | --             | 14.9          | 60.0     | 54.7                   |
| Formalin    | 1000                 | 15             | 24.0          | 45.5     | 53.2                   |
| Formalin    | 1000                 | 60             | 10.8          | 29.5     | 62.3                   |
| Formalin    | 1667                 | 15             | 38.3          | 20.5     | 63.5                   |
| Formalin    | 1667                 | 60             | 10.5          | 0.5      | 77.0                   |
| Formalin    | 2000                 | 15             | 38.5          | 7.3      | 69.4                   |
| Formalin    | 2000                 | 60             | 38.5          | 10.7     | 74.9                   |

<sup>a</sup>Hatch calculation:  $\frac{\text{number hatched}}{\text{total} - \text{initial morts}} \times 100$

Appendix 7. Hatching success (%)<sup>a</sup> of rainbow trout green and eyed eggs after dip treatments using candidate fungicide compounds.

| Chemical     | Egg stage | Number of dips | Duration of dips (min) | Concentration mg/L |            |            |            |              |
|--------------|-----------|----------------|------------------------|--------------------|------------|------------|------------|--------------|
|              |           |                |                        | 0                  | 2.5        | 5          | 15         | 25           |
| A-39909      | Green     | 6              | 15                     | 76                 |            |            |            | 0            |
|              | Green     | 6              | 60                     | 76                 | 58.30      | 0          | 0          | 0            |
|              | Eyed      | 5              | 15                     | 82                 | 90         | 84         | 8          | 0            |
|              | Eyed      | 5              | 60                     | 86                 | 94         | 0          | 0          | 0            |
|              |           |                |                        | <u>0</u>           | <u>2</u>   | <u>4</u>   | <u>12</u>  | <u>20</u>    |
| A-73336      | Green     | 6              | 15                     | 78                 | 84         | 92         | 90         | 86           |
|              | Green     | 6              | 60                     | 84                 | 82         | 84         | 98         | 84           |
|              | Eyed      | 6              | 15                     | 100                | 96         | 94         | 98         | 98           |
|              | Eyed      | 6              | 60                     | 96                 | 96         | 98         | 96         | 100          |
|              |           |                |                        | <u>0</u>           | <u>1</u>   | <u>2</u>   | <u>6</u>   | <u>10</u>    |
| Dichlorophen | Green     | 6              | 15                     | 68                 |            |            |            |              |
|              | Green     | 6              | 60                     | 80                 | 62.68      | 62.76      | 44.58      | 62.4         |
|              | Eyed      | 4              | 15                     | 100                | 96         | 92         | 96         | 100          |
|              | Eyed      | 4              | 60                     | 96                 | 96         | 98         | 92         | 86           |
|              |           |                |                        | <u>0</u>           | <u>50</u>  | <u>100</u> | <u>300</u> | <u>500</u>   |
| Diquat       | Green     | 6              | 15                     | 86                 | 82         | 88         | 86         | 90           |
|              | Green     | 6              | 60                     | 88                 | 88         | 86         | 94         | 90           |
|              | Eyed      | 6              | 15                     | 92                 | 98         | 96         | 90         | 92           |
|              | Eyed      | 6              | 60                     | 94                 | 92         | 94         | 96         | 94           |
|              |           |                |                        | <u>0</u>           | <u>100</u> | <u>250</u> | <u>750</u> | <u>1,250</u> |
| Formalin     | Green     | 6              | 15                     |                    |            |            |            | 64           |
|              | Green     | 6              | 60                     | 50                 | 52         | 80         | 80         | 82           |
|              | Eyed      | 3              | 15                     | 86                 | 86         | 80         | 80         | 64           |
|              | Eyed      | 3              | 60                     | 74                 | 74         | 72         | 36         | 60           |

Appendix 7. Continued.

| Chemical                   | Egg stage | Number of dips | Duration of dips (min) | Concentration mg/L |            |           |            |            |
|----------------------------|-----------|----------------|------------------------|--------------------|------------|-----------|------------|------------|
|                            |           |                |                        | 0                  | 5          | 10        | 30         | 50         |
| Herbisan                   | Green     | 6              | 15                     | 26                 | 22         | 58        | 56         | 34         |
|                            | Green     | 6              | 60                     | 46                 | 14         | 36        | 28         | 0          |
|                            | Eyed      | 3              | 15                     | 42                 | 64         | 54        | 70         | 52         |
|                            | Eyed      | 3              | 60                     | 36                 | 44         | 58        | 46         | 38         |
|                            |           |                |                        | <u>0</u>           | <u>25</u>  | <u>50</u> | <u>150</u> | <u>250</u> |
| KMnO <sub>4</sub>          | Green     | 6              | 15                     | 80                 | 78         | 66        | 70         | 64         |
|                            | Green     | 6              | 60                     | 80                 | 70         | 88        | 54         | 10         |
|                            | Eyed      | 3              | 15                     | 62                 | 62         | 80        | 86         | 68         |
|                            | Eyed      | 3              | 60                     | 60                 | 54         | 58        | 62         | 8          |
|                            |           |                |                        | <u>0</u>           | <u>2.5</u> | <u>5</u>  | <u>15</u>  | <u>25</u>  |
| Malachite green            | Green     | 6              | 15                     | 70                 | 76         | 72        | 78         | 80         |
|                            | Green     | 6              | 60                     | 68                 | 60         | 74        | 76         | 0          |
|                            | Eyed      | 5              | 15                     | 86                 | 96         | 86        | 86         | 90         |
|                            | Eyed      | 5              | 60                     | 86                 | 94         | 92        | 90         | 54         |
|                            |           |                |                        | <u>0</u>           | <u>25</u>  | <u>50</u> | <u>150</u> | <u>250</u> |
| Quinolinol SO <sub>4</sub> | Green     | 6              | 15                     | 58                 | 50         | 20        | 12         | 6          |
|                            | Green     | 6              | 60                     | 74                 | 6          | 0         | 0          | 0          |
|                            | Eyed      | 4              | 15                     | 98                 | 92         | 96        | 96         | 94         |
|                            | Eyed      | 4              | 60                     | 94                 | 94         | 94        | 92         | 64         |

<sup>a</sup>Each concentration was tested on 25 eggs per jar with each treatment concentration replicated (n=50).